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Dissertation for MSc degree on Biomedical Sciences, presented to the University of the Azores

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"Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time."

Thomas A. Edison

"Sometimes we want what we want
even if we know it's going to kill us."

Donna Tartt, *The Goldfinch*

To my parents and my beloved deceaseds…

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ABSTRACT

Repeat expansion disorders are caused by unstable expansion of repeat motifs, which can be pathogenic when a normal range is exceed. Spinocerebellar ataxias (SCAs) are autosomal dominant cerebellar ataxias with an adult-onset and a sub-group of repeat expansion disorders which is caused by triplet repeats. Machado-Joseph disease (MJD), or spinocerebellar ataxia type 3 (SCA3), is an autosomal dominant neurodegenerative disorder caused by a CAG expansion, belonging to the group of polyglutamine disorders. The expanded CAGs occurs in the *ATXN3* gene, at 14q32.1 (normal alleles - 12 to 44 CAG repeats, mutated alleles - above 52 CAG repeats). This late onset disorder, the most common from its group, presents a high prevalence in the Azores, especially in Flores Island. MJD molecular testing is based on PCR and automated capillary electrophoresis. However, in the presence of an apparently normal homozygous result, PCR might not be conclusive, since expanded alleles might fail amplification. In such cases, complementary techniques, as Southern Blot or Triplet Repeat Primed PCR (TP-PCR), should be applied. The purpose of this study was to develop and validate a TP-PCR protocol for MJD. Sixty-six blood samples previously genotyped by PCR were used to optimize and validate a TP-PCR assay. The robustness of the technique was evaluated using 14 buccal swab samples of heterozygous patients carrying an expanded allele. TP-PCR results were concordant with the ones obtained by PCR for 65 blood samples. The results obtained for the buccal swabs samples were fully concordant with the ones obtained for blood samples showing that the technique can be applied to different tissues and with low amounts of DNA. The TP-PCR developed and validated for MJD

should constitute a reliable complementary technique in diagnostic laboratories to overcome the limitations of standard PCR related with the failure in amplification of the expanded alleles.

RESUMO

As doenças de expansão são causadas pelo elevado número de motivos de repetição instáveis, que são patogénicos quando ultrapassam um valor normal. As ataxias espinocerebelosa (SCAs) são ataxias cerebelosas autossómicas dominantes com um início na idade adulta, constituindo um subgrupo das doenças de expansão que é causado por repetições de tripletos. A doença de Machado-Joseph (DMJ), ou ataxia espinocerebelosa do tipo 3 (SCA3) é uma doença neurodegenerativa autossómica dominante causada pela expansão de um motivo CAG e corresponde a doença de poliglutamina; a expansão ocorre no gene *ATXN3,* em 14q32.1 (alelos normais – 12 a 44 repetições CAG, alelos mutados – acima de 52 repetições CAG). Esta doença é a mais comum do seu grupo, e apresenta uma elevada prevalência nos Açores, especialmente na ilha das Flores. O teste molecular da DMJ baseia-se no uso da PCR, seguida de eletroforese capilar. Contudo, em indivíduos aparentemente homoalélicos para um alelo normal, a PCR pode ser inconclusiva, uma vez que os alelos maiores podem não ser amplificados constituindo um problema no teste molecular para a DMJ. Nestes casos devem ser aplicadas outras técnicas, como o "Southern Blot" ou a "Triplet Repeat Primed PCR" (TP-PCR). Este estudo teve como objetivo o desenvolvimento e a validação de um protocolo de TP-PCR para a DMJ. Sessenta e seis amostras de sangue previamente genotipadas por PCR foram utilizadas na otimização e validação da TP-PCR. A robustez da técnica foi testada em 14 amostras de esfregaço bucal de doentes heterozigóticos com um alelo expandido. Os resultados da TP-PCR foram concordantes com os obtidos pela PCR em 65 amostras de sangue. Os resultados obtidos para as amostras de esfregaço bucal foram 100% concordantes com os das amostras de sangue,

demostrando que a técnica pode ser aplicada em diferentes tecidos e para uma baixa quantidade de DNA. A TP-PCR desenvolvida e validada para a DMJ deverá constituir uma técnica complementar fiável em laboratórios de diagnóstico, de modo a ultrapassar as limitações da PCR, relacionadas com a falha de amplificação dos alelos expandidos.

CHAPTER 1. GENERAL INTRODUCTION

1.1. PERTINENCE AND PURPOSE OF THE WORK

Repeat expansion disorders are caused by unstable expansions of repeat motifs, usually triplets; when such repeat motifs exceed the normal range they originate disease (Harel *et al*., 2015).

Autosomal dominant cerebellar ataxias represent the group of spinocerebellar ataxias (SCAs) (Kim & Cho, 2015), which are considered adult-onset progressive neurodegenerative rare diseases (1 to 5 patients per 100000 people worldwide) (Ruano *et al*., 2014). Besides sharing a late age at onset, clinical features of these diseases overlap, transforming the diagnosis in a difficult process, which seems impossible to establish on clinical basis alone (Kim & Cho, 2015). Therefore, a molecular diagnosis is necessary to identify the different SCAs. SCAs can be classified in different groups according to the genetic mechanism of mutation; polyglutamine (polyQ) diseases are an important group of SCAs caused by an expansion of CAG repeat motifs in the coding region of the affected genes (Cortes & La Spada, 2015).

Machado-Joseph disease (MJD) also known as spinocerebellar ataxia type 3 (SCA3), is the second most common polyQ disease (Pringsheim *et al*., 2012). The *ATXN3* gene is located at 14q32.1 (Takiyama *et al*., 1993) and its normal alleles present from 12 to 44 CAG repeats, while expanded alelles consensually display above 52 repeats (reviewed in Bettencourt & Lima, 2011). CAG repeats in mutated *ATXN3* alleles tanslate into abnormally elongated polyQ tracts in the corresponding protein, ataxin-3 (reviewed in Durcan & Fon, 2013). This disorder, first described 43 years ago (Nakano *et al*., 1972), is the most prevalent disease from the group of

SCAs (de Castilhos *et al*., 2014; Ruano *et al*., 2014). MJD presents a high prevalence in Portugal, especially in Azores with a cluster in Flores Island, where the prevalence is 1 per 146 individuals (Araújo, 2012). This high prevalence justifies an investment on the development of a molecular technique that could be used to detect the mutation and to give a more precise diagnosis.

The localization of the *ATXN3* gene (Takiyama *et al*., 1993) and the identification and characterization of MJD's causative mutation (Kawaguchi *et al*., 1994) allowed the molecular testing of patients, with the aim of confirming a clinical diagnosis; moreover, it also allowed testing of healthy at risk individuals, in the context of pre-symptomatic, prenatal or pre-implantatory test (reviewed in Bettencourt & Lima, 2011). The best practice guidelines for molecular testing of SCAs, described by the European Molecular Genetics Quality Network (EMQN) (Sequeiros *et al*., 2010) established, among other aspects, the techniques used in molecular diagnosis. Routinely, molecular testing of polyQ SCAs is based on PCR using fluorescent-labeled primers, the DNA fragments being subsequently separated by automated capillary electrophoresis (CE). Such techniques, however, may not be conclusive in situations in which a sample appears to be homozygous for a normal allele, since large alleles can be difficult to amplify, leading to allelic dropout (Butler, 2012). In such cases, the possibility of non-amplification of the expanded allele must be excluded (Sequeiros *et al*., 2010). Southern Blot (SB) is proposed as one of the techniques which should be used for homoallelism confirmation (Sequeiros *et al.*, 2010); SB, however, is not cost effective, requires high concentrations of DNA and its time consuming (Trouba, 2005). Triplet Repeat Primed PCR (TP-PCR) is a complementary technique proposed for ambiguous cases

where non-amplification of the expanded allele needs to be ruled out (Sequeiros *et al.*, 2010). Standardized TP-PCR protocols have already been established and published for SCA2 (Cagnoli *et al*., 2006), SCA7 (Cagnoli *et al*., 2006), SCA8 (Krysa *et al.*, 2012), SCA10 (Cagnoli *et al.*, 2004), SCA12 (Cagnoli *et al*., 2004), Fragile X syndrome (Lyon *et al*., 2010; Seneca *et al*., 2012), Friedrich ataxia (Cagnoli *et al*., 2004), Huntington disease (Falk *et al*., 2006; Jama *et al*., 2013) and Myotonic dystrophy (Catalli *et al*., 2010; Singh *et al.*, 2014; Warner *et al*., 1996). For MJD, although TP-PCR is known to be implemented at some diagnostic laboratories, using in-house protocols, to the best of our knowledge, no validation report has been published. The main goal of this work was to develop and validate a TP-PCR protocol for MJD, which allows its standardized utilization in molecular diagnosis laboratories as a technique to identify the absence/presence of expanded alleles in homozygous samples.